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Downloading Central Clock Information in *Drosophila*

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Abstract

Pigment-dispèrsing factor (PDF) neuropeptide is an important neurochemical that carries circadian timing information originating from the central oscillator in *Drosophila*. Several core-clock factors function as upstream pdf regulators; the *dClock* and *cycle* genes control *pdf* transcription, whereas the *period* and *timeless* genes regulate post-translational processes of PDF via unknown mechanisms. For a downstream neural path, PDF most likely acts as a local modulator, which binds to its receptors that are possibly linked to Ras/MAPK signaling pathways. PDF receptor-containing cells seem to localize in the vicinity of nerve terminals from pace-making neurons. Although PDF is likely to be a principal clock-output factor, our recent evidence predicts the presence of other neuropeptides with rhythm-relevant functions. Furthermore, recent microarray screens have identified numerous potential clock-controlled genes, suggesting that diverse physiological processes might be affected by the biological clock system.

Index Entries: Circadian; clock; *Drosophila;* neuropeptide; output; pigment-dispersing factor.

Introduction

Photoperiodic changes influence life styles in most living organisms on Earth in such a way that, with rare exceptions, most animals have evolved to be active during night (nocturnal), day (diurnal), or at dawn and dusk (crepuscular). One of the major selective forces for such a distinct daily activity pattern exhibited by a given species is perhaps to avoid interspecific competition over limited natural resources, thereby maximizing their chances of survival.

Circadian behavioral rhythms in *Drosophila* are manifest in the adult locomotor activity and pupal-to-adult eclosion rhythms. Under light–dark (LD) cycles, wild-type flies show bimodal activity patterns (crepuscular), in which they exhibit peak activities during dark-to-light (i.e., morning) and light-to-dark transi-

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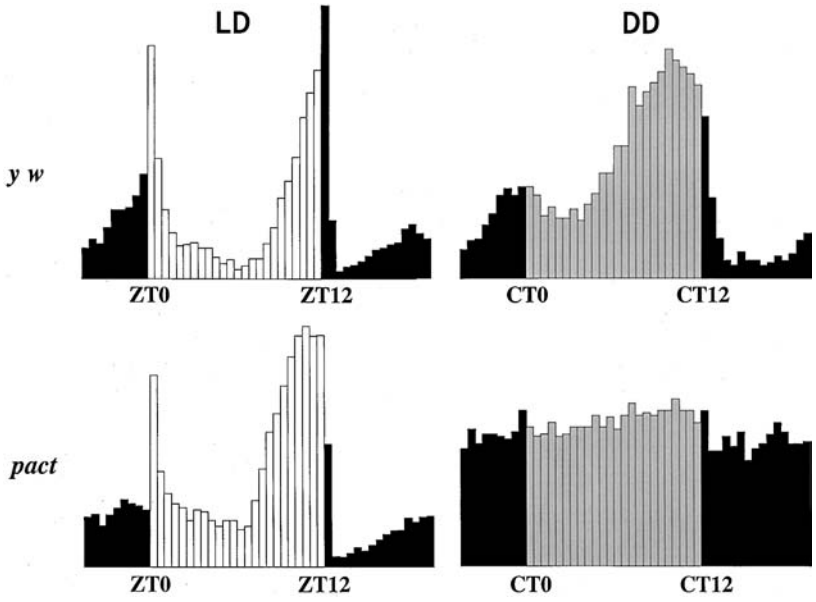

Fig. 1. Average locomotor activity rhythms. Control *y w* (*n* = 19) and mutant homozygous flies (*n* = 37) were entrained to 12-h-light:12-h-dark (LD) cycles for 7 d and subsequently proceeded to constant darkness (DD) for 9 d. This mutant gene is tentatively referred to as *pact* (an acronym derived from *pdf act*ivator). Data collection and analysis were performed as described *(1).* Each bar represents average activity for a half-hour period. Activities during day, night, or subjective day are indicated by white, black, or gray bars respectively. ZT-zeitgeber time (light-on at ZT0 and light-off at ZT12); CT-circadian time under DD condition. From the various independent behavioral assays, the authors have observed that 50–100% of the mutant flies were arrhythmic, whereas over 90% of *y w* control flies were rhythmic.

tions (i.e., evening), and they are relatively quiescent during midday and midnight (*1; see also* Fig. 1). When flies are subsequently transferred to constant darkness (DD), morning peaks gradually disappear, whereas evening peaks persist, suggesting that evening activity peaks are the authentic circadian component (Fig. 1); however, it has been postulated that the morning peaks are regulated by an independent oscillator *(2).* Nevertheless, continuation of the behavioral rhythms under constant environmental conditions suggests that the circadian rhythms are driven not just by external changes, but by internal clock activities.

The adult eclosion is a culmination of an insect development that occurs only once during the entire life cycle. Normally, it takes place during a specific time-window (or gate) of the day; those pupae who miss the gate have to wait for the next one to initiate eclosion processes, although the adult structures are already fully formed inside the pupal case *(3).* By comparison with rest–activity rhythm of an individual fly, eclosion rhythm is a population rhythm in which mixed-age populations of developing wild-type pupae raised under LD cycles molt to become adult flies with peaks soon after light-on. Furthermore, this rhythmic adult emergence persists under DD cycles with periodicity close to 24 h *(4).*

These behavioral rhythms are governed by biological clock system, which is conveniently categorized by sequential input pathway, pacemaker (or oscillator), and output pathway. External time cues (principally LD cycles) perceived by circadian photoreceptors are relayed

to the central oscillator (input pathway) where this timing information is stored, integrated and processed (pacemaker), and then delivered to peripheral targets (output pathway). Since the first clock locus *period (per)* was discovered in *Drosophila (5),* genetic screens for abnormal behavioral phenotypes have revealed several clock genes: *timeless (tim), dClock (dClk), cycle (cyc), double-time (dbt), cryptochrome (cry),* and *shaggy (sgg).* Orthologs of the fly clock genes have also been discovered in mammals, suggesting that fundamental molecular clock mechanisms are conserved throughout the long evolutionary path (for recent reviews, *see* refs. *6,7*). Further biochemical and molecular genetic characterizations revealed that these clock gene products interact to generate negative and positive molecular feedback loops, leading to the circadian oscillations of *per, tim,* and *dClk* gene products (for review, *see* ref. *8*). Since similar feedback loops are also essential for normal clock functions in other organisms (such as mouse and fungus), these autoregulatory loops might be the core elements for the molecular time-keeping mechanisms *(7).*

Despite extensive biochemical and genetic studies on details of the central clock function, we are still deficient in understanding the mechanisms by which these clockworks are decoded so that they regulate overt rhythmic phenotypes (i.e., output pathways). Because the clock operating system resides in the distinct clusters of neurons of the central brain *(9–11),* it is hard to imagine that it controls circadian phenotypes (e.g., locomotor activities) directly. Rather, pacemaker neurons may produce extracellular signals in a time-sensitive manner that deliver clock messages to the downstream targets (for instance, motor centers that regulate locomotor activity—such anatomical structures have not been defined), which in turn control daily rhythms more directly.

The candidate clock-output factors should meet the following criteria: 1) They must control circadian rhythms in which animals carrying loss-of-function and/or gain-of-function mutations in these output factor-encoding genes (or other genes whose products are crucial for biosynthesis of non-proteinaceous output factors [e.g., melatonin] or for secretion of such factors) exhibit abnormal rhythms. 2) The expression of these gene products and/or their post-transcriptional or post-translational processes should be controlled by core-clock regulators or their target gene products.

As the molecular genetic studies of clock-output pathways form a newly emerging subdiscipline in the area of chronobiology, the focus will primarily be on recent progresses in the clock-output mechanisms that might direct diverse physiological and behavioral rhythms in flies.

Brain-Derived Factors Involved in the Clock-Output Pathways

Novel brain transplantation experiments suggested the presence of brain-derived factors that are released from the pacemaker cells located in the brain. When the brain from the fast-clock mutant per^S––a *per* allele that produces free-running rhythms (tau) with approx 19-h period *(5)*––was transplanted into the abdomen of arrhythmic per^0 flies, the recipients restored behavioral rhythmicity characteristic of the brain donor (i.e., tau approx 19 h) *(12).* These studies strongly suggested that the brain derived-humoral factors controlled circadian locomotor activity rhythms.

Similarly, it was shown that circadian eclosion eclosion rhythms are also controlled by brain factors in the silkmoth *(13).* In this report, a surgical removal of brains from pupae (debrained) caused a random fashion of eclosion (i.e., arrhythmic); however, when debrained pupae were implanted with brains from other pupae which had been entrained to a certain LD regime, the recipient eclosed in a circadian fashion. Because adult ecdysis behavior is initiated and directed by a neuroendocrine cascade *(14),* these neuroendocrine factors are potentially under the clock regula-

tion. Taken together, these studies strongly argued for the presence of neurohumoral factors that relayed circadian messages from the central oscillator to peripheral tissues, thereby controlling overt behavioral rhythms in insects.

PDF, the First Clock Transmitter Identified

These studies suggest that clock-output pathways are mediated by humoral factors whose synthesis and/or release is regulated by the central oscillator. One such candidate molecule is a neuropeptide, termed pigment-dispersing factor (PDF). PDFs are insect neuropeptides that show structural homology to their crustacean counterparts, pigment-dispersing hormone (PDH), thereby forming a neuropeptide family in arthropods *(15).* In contrast to crustacean PDH, insect peptides have been referred to as pigment-dispersing "factor," since the hormonal functions of insect peptides had not been defined.

The clock-relevant functions of PDF in insects stem from the physiological actions of PDH in crustaceans: translocation of retinal distal pigment and epithelial chromatophoral pigment dispersion *(15).* These color changes take place in a circadian manner, and it was thus proposed that PDH is a primary effector for pigmentation rhythms in crustaceans *(16,17).* In contrast, circadian cuticular color changes are far less pronounced in insects, indicating different biological functions of PDF. Interestingly, brain lesion studies combined with immunocytochemistry and behavioral analyses in cockroaches found a positive correlation between the presence of PDF-immunoreactive neurons and normal circadian locomotor activity rhythms *(18).* Furthermore, PDF-immunoreactivities were detectable in a subset of *per*-expressing clock neurons in the *Drosophila* brain *(19).* These results provided an important clue for rhythm-relevant PDF functions in flies.

To delve into the roles of PDF for a clock transmitter, the fly PDF-encoding gene *(pdf)*––which was the first *pdf* gene characterized in insects *(20)*––was cloned. Subsequently discovery revealed a fortuitous *pdf*-null mutant (pdf^0) in which a base-substitution mutation spontaneously occurred to create a prematurely terminated PDF precursor. This resulted in the elimination of PDF-production *(11).* The locomotor activity rhythms of pdf^0 mutant flies are quasi-normal under 12-h-light: 12-h-dark cycles, except for little anticipation of light-on signal and earlier evening activity peak than wild-type one. When these flies are transferred to DD, the rhythmicity is gradually lost during the first 2–3 DD days, becoming arrhythmic. These aberrant pdf^0's behavioral phenotyes are sharply contrasted to other arrhythmic central clock mutant ones, in which they are poorly entrained to LD cycles and become immediately arrhythmic under subsequent DD conditions *(e.g., 21,22).*

A similar degree of abnormality in the behavioral rhythms exhibited by pdf^0 mutant is observed for the flies in which the *pdf*-expressing neurons are selectively ablated, suggesting that *pdf* neurons are at least part of circadian pacemaking cells in *Drosophila (11).* Since the adult eclosion rhythms are similarly abnormal in the pdf^0 mutant (F.R. Jackson, personal communication), PDF is likely to serve as a principal clock-output regulator for both behavioral rhythms in *Drosophila.*

Upstream of the *pdf* Gene

As was mentioned earlier, candidate clock-output factors should be regulated by central clockworks directly or indirectly; it was determined that *pdf* is indeed regulated by several clock genes at the transcriptional and post-translational levels *(23).*

Normally, *pdf* is expressed in a cluster of four neurons in each larval brain hemisphere, and these neurons persist during metamorphosis developing into the small ventrolateral neurons (s-LN_vs) in the adult brain (*see* Fig. 2).

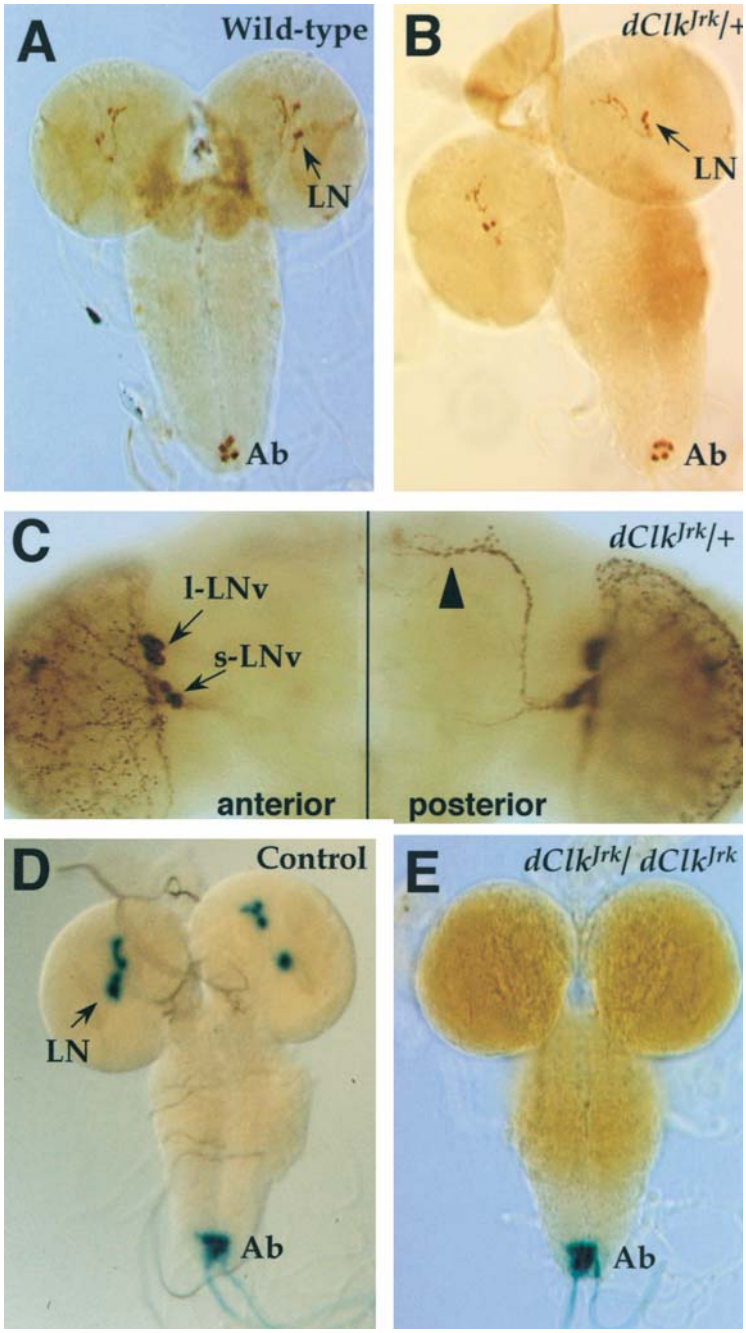

Fig. 2. *In situ* expression of the *pdf* gene in whole-mounted CNS. **(A–C)** PDF-immunohistochemistry. **(A)** Wild-type third-instar larval CNS. Normally PDF is expressed in four lateral neurons (LN) in each brain lobe, and 4–6 neurons in the abdominal ganglion (Ab). **(B)** $dClk^{Jrk}$/+ heterozygous third-instar larval CNS. These PDF-immunosignals are indistinguishable from those of wild-type. **(C)** PDF-immunoreactive neurons in the adult brain from $dClk^{Jrk}$/+ heterozygotes. These immunostaining patterns are also identical to those of wild-type adult brain (cf *23*). Two clusters of neurons (small-ventro-lateral neurons (s-LN_v) and large-ventro-lateral neurons (1-LN_v)) are clearly visible in the anterior view (left). In the posterior view (right), dorsal projection from s-LN_vs is indicated by an arrowhead and is essential for normal circadian behavioral rhythms, as described in the text *(23,25,26)*. **(D,E)** *pdf*-promoter driven *lacZ* reporter expression detected by X-gal histochemistry of the third-instar larval CNS. **(D)** control. The *lacZ* gene expression patterns are identical to those observed by PDF-immunohistochemistry shown in **(A). (E)** $dClk^{Jrk}/dClk^{Jrk}$ homozygotes. Consistent with our previous *pdf in situ* hybridization and immunohistochemistry results reported else *(23), pdf* promoter-mediated *lacZ* expression is absented only from the lateral neurons. At least five brain specimens were processed and examined for each panel.

Nerve fibers stemming from these cells project into the superior protocerebrum near mushroom body calyxes, and these neuronal processes are essential for normal rhythmicity *(24–26).* In the vicinity of s-LN_v perikarya, another cluster of four *pdf*-expressing neurons with larger somata (thus referred to as large ventrolateral neurons, 1-LN_vs) emerge during metamorphosis. These cells project ipsilaterally and contralaterally into the medulla cortex (Fig. 2; *see* also ref. *24*).

pdf mRNA levels and PDF-immunoreactivities are severely reduced or eliminated in arrhythmic cyc^0 or $dClk^{Jrk}$ mutant brains, respectively, suggesting that *pdf* transcription is positively controlled by these clock transcription factors *(23; see* also Fig. 2). Interestingly, *in situ pdf* expression reduction in these mutant backgrounds is observed primarily in lateral neurons (LNs) in the larval *(23,27)* and their descendent s-LN_vs in the adult central nervous system (CNS) *(23).* These results were further confirmed by *pdf* promoter-driven *lacZ* reporter gene expression in which the $dClk^{Jrk}$ larval CNS showed complete absence of *lacZ* expression in the LNs (Fig. 2).

How do these clock transcriptional regulators activate *pdf* expression? It is a well known fact that dCLK:CYC heterodimer is a functional unit that activates transcription of target genes via binding to the consensus 6-bp E-box (CACGTG) sequence present in these target gene promoters *(27–31).* Despite the presence of E-box sequence in the *pdf* promoter, it is definitely not involved in *pdf* transcriptional regulation, suggesting that *pdf* is activated by dCLK and CYC in an indirect manner *(23).* In addition, if the *pdf* gene is a direct target for dCLK:CYC heterodimer, *pdf* transcript levels should oscillate in phase with *dClk* mRNA *(32).* However, the lack of *pdf* mRNA rhythms *(20)* does not support direct regulation of *pdf* by the *dClk* and *cyc* gene products. Another argument for indirect regulation comes from the observation of differential effects of $dClk^{Jrk}$ and cyc^0 mutations on *pdf* transcription as descrined earlier *(23).* If *pdf* transcription were directly activated by dCLK:CYC complexes, the absence of either factor should produce the same results. Having said this, $dClk^{Jrk}$ is most likely to be a *dClk*-null allele, as the EMS-induced non-sense mutation eliminates most of the glutamine-rich transcriptional activation domain of the dCLK protein *(21).*

On the basis of these observations, the authors have postulated the presence of a third factor that may function as a direct activator for *pdf* transcription. Alternatively, this hypothetical factor may be complexed with dCLK and CYC proteins to form the complete *pdf* transcriptional machinery. If this were true, then a hypomorphic (or amorphic) mutation in this putative transcription factor-encoding gene would reduce (or eliminate) *pdf* expression. In accord with this prediction, the authors recently found a *P*-transposable element insertion line in which *pdf* mRNA and peptide were barely detectable in the larval LNs (J. Paik, G. Lee, J.H. Park, unpublished results). Intriguingly, consistent with the histological defects, the mutant homozygous flies exhibited aberrant locomotor activity rhythms comparable to the pdf^0 mutant (Fig. 1; cf ref. *11*). Molecular characterizations of the gene disrupted by the *P*-element insertion are currently under investigation.

It should also be noted that PDF-immunosignals are normal in the CNS of the $dClk^{Jrk}$ heterozygotes (Fig. 2). These results are contrasted to the behavioral phenotypes in which the $dClk^{Jrk}$ mutant allele acts as a semidominant for the locomotor activity rhythms *(21).* Perhaps, dCLK proteins have dual functions in a distinctive manner-one for central clockworks that require a full dose of dCLK proteins, and the other for the downstream gene regulation for which only a half dose of dCLK is sufficient. In this regard, it would be interesting to see if $dClk^{Jrk}$ mutation also acts on other *dClk*-regulated genes in a recessive manner.

pdf is also regulated post-translationally by separate clock genes. A lack of *pdf* mRNA cycling was previously reported *(20);* however, we observed that PDF-immunoreactivities at the axon terminals stemming from the s-LN_vs

change circadianly in wild-type *(23,26).* Therefore, the authors speculate that circadian variations of PDF content in the nerve terminals may reflect the periodic secretion of PDF. More importantly, this cycling occurs with a short period (approx 19 h) in per^S mutant, coincident with the behavioral periodicity of this mutant, and it is eliminated in arrhythmic per^0 and tim^0 mutants. These results led to the proposition that circadian PDF-release from the nerve terminals might be regulated by *per* and *tim.*

Although *per* and *tim* do not affect *pdf* mRNA levels *(20,23),* one possibility for the *per/tim*-regulated periodic PDF release is that *per* and *tim* may control the circadian excitability of *pdf*-lateral neurons. In line with this, mammalian pacemaker neurons exhibit autonomous circadian rhythms in the spontaneous firing rate, a parallel to the free-running period of locomotor activity rhythms *(33,34).* Whether similar electrophysiological activity rhythms are present in fly clock-neurons has yet to be tested.

Is PDF solely an output factor of the core-clock regulators? Perhaps so, as TIM oscillations in the LNs of the larval brain were normal in pdf^0 mutant; these results suggest that the central oscillator operates in a normal fashion in the absence of PDF *(35).* In opposition to this, one study indicates that PDF may also involve the entrainment pathways at least in cockroach, since the exogenous supply of PDF peptide near the pacemaker cells in the cockroach brain causes time-dependent phase delays of the locomotor activity rhythms *(36).* However, it is not known whether this type of neurochemical perturbation occurs under natural conditions. This does not seem to be the case in *Drosophila* because pacemaking s-LN_vs are not likely to be innervated by other PDFergic neurons (Fig. 2).

Downstream of the *pdf* Gene

Although PDF is an essential clock-output messenger in *Drosophila,* it is unknown where the PDF signal is transmited to. Circumstantial evidence indicates that PDF is likely to be a local-acting neuromodulator. For instance, although most of *disconnected (disco)* mutant flies tested are arrhythmic, very few of them (approx 1%) exhibit rhythmic behavior; their rhythmicity is correlated with the presence of residual PDF-immunoreactive neurons that project into the superior protocerebrum where s-LN_vs are normally arborized (*25; see* also Fig. 2). Moreover, transgenic misexpression of *pdf* in the neuronal cells that project into this area causes abnormal behavioral rhythms, perhaps by producing conflicting output signals *(26).* These studies strongly suggest that superior protocerebrum near mushroom body calyxes is a potential post-synaptic target area for PDF signaling pathways.

As for PDF receptive cells, they should express the membrane receptors for PDF binding and subsequent activation of signal transduction cascade. Although PDF receptors have not yet been identified, recent data suggest that PDF:receptor interactions may involve Neurofibromatosis-1 (Nf-1) and Ras/MAPK signaling pathway *(37).* Human Nf-1 proteins are known to function as a tumor suppressor by inactivating Ras function via Ras-specific GTPase-activating protein activities *(38).* In contrast, the fly homolog of Nf-1 seems to interact with cAMP signaling pathways to regulate overall body size, since dwarfism caused by *Nf-1* null mutation is rescued by expression of active cAMP-dependent protein kinase *(39).*

In addition to the body size, the *Nf-1* gene apparently plays a role in the clock-output pathways, since dwarf *Nf-1*-null mutant flies show arrhythmic free-running activity rhythms, but central clock in this mutant background operates normally *(37).* The abnormal circadian behavior of the *Nf-1* mutant was partially rescued by loss-of-function mutations in Ras/MAPK signaling pathways, suggesting that the circadian role of *Nf-1* is mediated by Ras/MAPK pathways. Furthermore, levels of activated MAPK (i.e., phosphorylated MAPK) oscillate in the vicinity of axon terminals of s-LN_vs and decrease in pdf^0 mutant background. Perhaps, arrhythmic free-running behavior

displayed by *pdf*^{0} mutant flies could at least be partially due to the failure of MAPK activation. These results, together with our previous data, argue strongly for a hypothesis in which rhythmically secreted PDFs from s-LN$_v$s bind to their cell-surface receptors localized in the superior protocerebral neurons which in turn activate the Ras/MAPK signal transduction pathways.

If the above hypothesis is true, then what type of molecules would serve for the PDF receptor? It is generally agreed upon that neuropeptide ligands bind to the heteromeric G-protein coupled receptors (GPCR; e.g., ref. *40*), which typically activate cAMP or inositol trisphosphate/Ca^{2+} signaling pathways. By comparison, Ras/MAPK signal transduction is activated primarily by receptor tyrosine kinases (RTKs), such as Torso and the epidermal growth factor (EGF) receptor homolog, which are involved in various cell-fate determination (for review, *see* ref. *41*). Inferred from the potential involvement of Ras/MAPK in the PDF signal transduction, it is possible that the PDF receptor belongs to the RTK family rather than GPCR family. However, it is similarly plausible that the PDF receptor is a member of the GPCR family which may activate Ras/MAPK via cross-talk between different signaling pathways, since transactivation of multiple signaling pathways for a given peptide ligand is not uncommon (for review, *see* ref. *42*). Molecular characterizations of the PDF receptor will clarify this issue.

Of a great interest, a member of RTK family is also involved in the mammalian locomotor activity rhythms. Kramer et al. *(43)* recently identified the transforming growth factor-α (TGF-α) as a "locomotor inhibitory factor" secreted from the suprachiasmatic nucleus (SCN) of the hypothalamus—the mammalian pacemaker. Further evidence indicated that such inhibitory action of TGF-α seemed to be mediated by EGF receptor signaling. These studies, together with works in flies, reiterate the importance of RTK and subsequent Ras/MAPK signal transduction cascade for clock downstream.

Is PDF the Only Neurochemical Controlling Locomotor Activity Rhythms?

The answer to this question is simply "no" owing to the following evidence. First, not all *pdf*-null mutant flies show arrhythmic behavior. Depending on the genetic background, it is routinely observed that 50–98% of *pdf*^{0} flies show abnormal free-running activity rhythms *(11).* In contrast, flies carrying null-mutations in the central clock genes under any genetic backgrounds show almost complete arrhythmicity, which strongly indicates that PDF may not be a sole clock mediator.

Second, *disco* mutant flies exhibit a more severe deficit in the locomotor activity rhythms than do *pdf*^{0} mutant *(11).* The *disco* mutation causes abnormal brain structures in which photoreceptor cells are disconnected from the optic lobes, and it disrupts normal development of the optic ganglia *(44).* Interestingly, two clusters of *per*-expressing lateral neurons (more dorsally located lateral neurons [termed LN$_d$s] plus ventrally located *pdf*-expressing s- and l-LN$_v$s) are missing in the *disco* mutant brain *(45),* indicating a role for LN$_d$s in the clock function. This is further supported by our previous data showing that a selective ablation of *pdf*-neurons (i.e., s-LN$_v$s + l-LN$_v$s) causes less severe behavioral defects than *disco* mutation does *(11).* Therefore, the residual rhythmicity could be due to the intact LN$_d$s in these transgenically manipulated flies. Another cellular component for clock system is dorsally located *per*-expressing neurons (DNs) in the adult brain *(46).* Somata of a subset of DNs are located near axon terminals stemming from pacemaking s-LN$_v$s, indicating that these cells are potentially post-synaptic targets for s-LN$_v$s. Given the putative rhythm-relevant functions for DNs and LN$_d$s, it is important to determine their neurotransmitter phenotypes, because these neurochemicals are possibly involved in the clock-output signal cascade.

Third, disruption of synaptic transmission by ectopic tetanus toxin (TNT) expression in

the *per*- and *tim*-expressing cells caused abnormal activity rhythms, whereas more restricted TNT expression to the *pdf*-neurons did not impair them *(47,48).* These results indicate two things: 1), PDF-containing vesicle release does not involve synaptobrevin-dependent exocytosis. 2). Neurotransmitters present in non-PDF clock neurons, whose releases are prevented by TNT, play roles in the regulation of locomotor activity rhythms.

Finally, the aforementioned brain-transplantation experiments predict the existence of neuro-humoral output factors that are supposedly secreted into the circulatory system *(12).* These factors are unlikely to be PDF, because circumstantial evidence indicates that PDF acts on post-synaptic neurons in the brain to transmit clock-relevant information *(25,37).* Perhaps PDF functions as a primary upstream output factor, modulating release of the secondary neurochemicals into the circulatory system, which in turn regulates overt circadian behaviors.

The authors recently presented more direct evidence to show the presence of other neuropeptides involved in the rest-activity rhythms *(49).* A number of insect neuropeptides are modified at their *C*-termini with α-amidation, which is important for their bioactivities. One of the enzymes that catalyzes α-amidation is the peptidylglycine α-hydroxylating monooxygenase (PHM), and null mutations in the *Drosophila* PHM-encoding gene cause lethality during embryonic development *(50).* When *PHM* is ectopically expressed in the subset of peptidergic neurons (including *pdf*-neurons) by a combination of enhancer-trap gal4 and *pdf*-gal4 drivers with UAS-*PHM,* lethality of the *PHM* mutant is rescued. However, these live adult flies are largely arrhythmic *(49).* Broader *PHM* expression in the clock cells directed by a *tim*-gal4 driver combined with the above gal4 transgenic lines almost completely restores arrhythmicity, suggesting that α-amidated neuropeptides other than PDF also play roles in the clock downstream pathway.

In congruence with the above results, it is notable that two neuropeptide genes (*corazonin* and *capability*) have been identified as negatively regulated by dCLK from the microarray screen, implying that these neuropeptides may have rhythm-relevant functions in flies *(51).* Corazonin is an α-amidated neuropeptide discovered originally in cockroaches as a potent heart beat stimulator *(52);* it does, however, seems to have species-specific functions as it lacks cardiostimulatory activities in other insect species, but instead causes dark pigmentation in locusts *(53,54).* The *corazonin*-encoding gene was cloned in *Drosophila* many years ago *(55),* but its function in flies remains elusive. In contradiction to the previous microarray results, however, our preliminary data indicate that the intensity and location of *in situ corazonin*-signals in $dClk^{Jrk}$ mutant CNS are comparable to those in wild-type (J.H. Park and G. Lee, unpublished data). Therefore, transcriptional regulation of the *corazonin* gene by *dClk* is questionable.

The *capability (capa)* gene most likely encodes a neuropeptide precursor which produces at least three putative neuropeptides via post-translational processing *(56).* Two of these are peptides homologous to cardioacceleratory peptide 2b (CAP2b), which was originally purified from the tobacco hornworm, *Manduca sexta (57).* In *Drosophila,* CAP2b stimulates the heart rate and fluid secretion by Malpighian tubules via the cGMP-signaling pathways *(58).* Nevertheless, as performed for the *corazonin* gene, further histochemical analyses for *capa* gene expression is necessary to confirm the microarray results.

Interestingly, *Drosophila* Malpighian tubules have their own clock, because free-running *per* and *tim* cyclings were observed in this tissue cultured in vitro *(59).* Physiological significance of this observation remains enigmatic, although one can speculate that this autonomous clock regulates the circadian fluid secretion rhythms. However, this could be further complicated by the fact that the excretory activities of Malpighian tubules are primarily regulated by several neuroendocrine factors (e.g., ref. *60*), whose releases may respond, not to circadian signals but, to particular physiological demands.

In summary, there seems to be more than one neuropeptide involving clock-output functions. As previously shown for the *pdf* gene, molecular identification and characterizations of these neuropeptide-encoding genes, their expression patterns, and effects of genetic manipulations of these genes on the rhythm-relevant phenotypes will certainly broaden our understanding of the regulatory roles of the neural substrates underlying clock-output pathways, and cellular components that form a neural network for clock information flow.

Genes that Are Involved in the Clock Output Pathways

In addition to the *pdf* gene, other clock-regulated genes have been discovered as is summarized below. Although none of these genes is a neuropeptide gene, some of them have a potential connection to *pdf* gene regulation. Others are likely to be involved in separate output arms of the central clockworks.

Vrille (vri)

The *vri* gene was identified by a differential display of genes expressed in wild-type adult heads at different times of the day, and its putative protein product is a member of the basic zipper (bZIP) transcription factor family *(27).* Rhythmic transcription of *vri* is in phase with *per* and *tim,* and is directly activated by dCLK and CYC via an E-box present in the promoter. However, *vri* mRNA oscillations are dampened in per^0 or tim^0 mutants at its intermediate or peak levels, respectively, indicating that *vri* transcription is regulated differentially by separate clock genes.

Constant high doses of *vri* alter behavioral rhythms and suppress *per* and *tim* expression; these results indicate that VRI acts as a negative regulator for *per* and *tim* transcription perhaps via interference with dCLK:CYC heteromeric activator *(27).* Direct physical interaction between VRI and dCLK:CYC is perhaps the simplest model to account for this molecular phenotype, but it needs to await biochemical data. In addition to the effects on *per* and *tim,* constitutive overexpression of *vri* also suppresses PDF peptide levels (but not *pdf* mRNA levels) in the larval lateral neurons, implying complex roles of *vri* in the circadian context *(27).*

Since *vri*-null mutants are lethal during embryonic development, *vri* is a vital gene *(61).* However, its role in the clock function of adult flies provides an example of pleiotropic gene functions at different life stages. Taken together, *vri* is apparently a clock-controlled gene which also participates in the central clockwork by acting as a negative regulator.

Related to *vri* is the *pdp1* gene which encodes a PAR (Proline and Acidic amino acid-Rich) activation domain-containing bZIP transcription factor. Moreover, *pdp1* mRNA levels cycle in wild-type and its transcription is directly regulated by dCLK *(51),* suggesting that *pdp1* might also be involved in the clock-output system in flies. Therefore, as is the case for *vri,* it will be interesting to determine whether *pdp1* regulates PDF production and other clock gene activities.

Intriguingly, mammalian bZIP transcription factors, E4BP4 and PAR-containing proteins, are structurally related to VRI and PDP1 respectively. As with VRI, E4BP4 functions as a transcriptional repressor and plays a role in the regulation of central clockworks. Recent evidence showed the circadian oscillations of *e4bp4* mRNA and protein levels in the SCN; it has therefore been postulated that *e4bp4* is a target gene for CLK:BMAL1 heterodimer (CLK and BMAL1 are mammalian homologs of dCLK and CYC respectively; *see* ref. *7*) *(62).* Like VRI, in vitro assays support the role of E4BP4 as a negative regulator, since it down-regulates the *Per2* gene in chicken *(63)* and the *Per1* gene in mouse *(62).* However, circadian behavioral phenotypes of *e4bp4*-deficient mouse has not yet been determined.

Three PAR transcription factors––hepatic leukemia factor (HLF), thyrotroph embryonic factor (TEF), and albumin *D*-element *b*inding

*p*rotein (DBP)––are the mammalian homolog of PDP1, and show robust daily rhythms in the SCN and the liver of mouse with phases opposite to those of *e4bp4* oscillations *(62).* Genetic ablation of the *dbp* gene in mice causes approx 0.5 h shorter free-running period than control, indicating its role in the clock function *(64).* Furthermore, *dbp* transcription is directly activated by CLK:BMAL1 via E-boxes located in the intronic sequences, verifying that *dbp* is a clock-controlled gene *(65,66).* However, DBP also functions as an activator for *mPer1* transcription via interaction with consensus DBP-binding site in the *mPer1* promoter, suggesting a role for *dbp* in the central clockwork as well *(66).* Circadian roles of the *hlf* and *tef* genes, albeit their molecular rhythms, are currently unknown, although it has been proposed that anti-phased oscillations of E4BP4 and PAR-proteins regulate molecular rhythms of their target genes by alternatively activating (PAR-proteins) or repressing (E4BP4) such genes at different times of the day *(62).*

The best-characterized target genes for *dbp* are several members of the cytochrome P450 family in the mouse liver, which may play roles in the regulation of circadian metabolic activities in this tissue (e.g., *67*). However, there is no direct output gene targeted by these transcription factors yet identified in the central oscillator in both mouse and fly, although VRI is likely to regulate PDF levels in an indirect manner *(27).* Hence, it is still unclear how these clock-controlled transcription factors are associated with the overt circadian behavioral rhythms.

Drosophila cAMP Response Element Binding Protein 2 (dCREB2)

Luciferase activities in CRE-luciferase (CRE-luc) transgenic flies vary in a circadian manner and are flat in the *dCREB2* mutant background, reflecting circadian changes in the dCREB2 activities *(68).* The role for dCREB2 in the clock regulation is further supported by the abnormal locomotor activity rhythms of *dCREB2* mutant flies.

dCREB2 activity is a *per*-downstream target since temporal expression patterns of CRE-luc reporter mirrored *per* mutations (i.e., no cycle in *per*-null, shorter rhythms in *per*-short, and longer rhythms in *per*-long mutant) *(68).* Unlike circadian cycling of dCREB2 activities, CREB2 protein levels (or its phosphorylation status) do not show apparent cycling, implying that circadian fluctuations in dCREB2 activities could be from the *per*-regulated cyclic expression of an unknown dCREB2 partner.

As is the case for *vri,* dCREB2 activity is likely to be involved in the central clock functions, since the amplitude of PER protein oscillations is lowered and *per*-luc reporter cycling is dampened in *dCREB2* mutant background *(68).* Nevertheless, together with the aforementioned Ras/MAPK signaling cascade, these studies provide us with more complicated multi-directional features for clock-output signaling mechanisms.

The roles of cAMP secondary messenger in the clock regulation is further strengthened by abnormal free-running activity rhythms of flies with altered cAMP levels, which are caused by mutations in the *dunce* (encodes the cAMP-specific phosphodiesterase) or *DC0* (encodes the catalytic subunit of cAMP-dependent protein kinase) loci *(69).* Since cAMP is produced by activation of trimeric G-protein coupled receptors, molecular identification of these receptors and their counterparts (i.e., ligands, perhaps neuropeptides) will provide important clues about the molecular and cellular components of clock-output pathway.

cAMP is apparently a key second messenger for olfactory learning and memory in flies, as the *dunce* and *DC0* mutant flies (and other mutants in which cAMP metabolism or signaling is altered) are defective in this type of behavior (for review, *see* ref. *70*). In addition, cAMP-signaling plays a role in the homeostatic regulation of "rest rebound" (rest- or sleep-deprivation during a day causes longer duration of rest in the next day) in *Drosophila (71).* Genetic manipulations that lead to reduction in cAMP levels or dCREB activities cause longer rest duration than wild-type control

does after its deprivation, without affecting normal clock functions (for recent review regarding sleep-like state in *Drosophila, see* refs. *72,73*). Considering these results jointly indicates multifunctional roles of cAMP-signaling pathways in diverse physiological processes in *Drosophila.*

Lark

The *lark* locus is involved in the regulation of circadian eclosion rhythms but not the locomotor activity rhythms *(74).* These results clearly demonstrate that different behavioral rhythms are independently regulated by the central clock. The *lark* gene encodes an RNA-binding protein whose abundance varies circadianly in wild-type pupae (pharate adults), but is flat in per^0 mutant *(75).* Moreover, LARK-immunoreactivities cycle in a defined set of peptidergic neurons which produce fly homolog of CCAP (*c*rustacean *c*ardio*a*ctive *p*eptide) *(76).* Previous studies in other insects indicate that CCAP plays a role as the terminal regulatory neuropeptide in the neuroendocrine hierarchy mediating adult ecdysis *(14).* Based on these observations, it has been proposed that LARK is involved in the circadian CCAP release or CCAP-neuron excitability, thereby controlling rhythmic eclosion *(76).*

To determine the roles of these peptidergic neurons for adult ecdysis in *Drosophila,* the authors recently carried out targeted CCAP-cell ablation using *CCAP* gene promoter; however, this led to lethality during pupal development (Park et al., in preparation). Thus, the physiological functions of CCAP-neurons (or CCAP *per se*) in the regulation of circadian ecdysis rhythm is currently unknown.

Take-Out (to)

The *to* gene has been identified by subtractive screening as a clock-downstream target. *to* encodes a secretory protein that is structurally similar to the juvenile hormone binding proteins *(77). to* mRNA and protein levels oscillate circadianly and are down-regulated in the clock mutant backgrounds, which places *to* in the output pathways *(78).* The canonical target sequence (E-box) for dCLK:CYC-mediated transcriptional activation is present in the *to* promoter; as is the case for the *pdf* gene, this E-box apparently is not employed as a *cis*-acting regulatory element, suggesting an indirect regulation of *to* by dCLK:CYC.

to mutant flies show normal circadian behaviors. Interestingly, however, the *to* gene products are induced by starvation, and *to* mutants are more prone to death than are wild-type upon starvation, indicating a role for *to* in the control of feeding and/or metabolic activities *(77).* Although diurnal feeding rhythms are not known for *D. melanogaster,* recent evidence shows that feeding activities are regulated by a clock gene in mammals, since inter-meal intervals become shortened in the clock mutant homozygous for the *tau* gene in hamster; this agrees with the short free-running period of locomotor activity rhythms exhibited by *tau* homozygous mutant hamsters *(79).* It is certainly possible that feeding rhythms are another tributary of the clock-output pathways in flies as well, since the fly-homolog *(double-time)* of the *tau* gene also functions in the central clockwork *(80).*

Recently, five genes related to the *to* were found to be clustered in the fly genome, forming a *to* gene family *(51).* Despite their molecular cyclings, the responses to dCLK seem to be variable, as some of them are up-regulated while others are down-regulated in $dClk^{Jrk}$ mutant background. Further genetic dissections of these genes are necessary to uncover their biological functions.

Drosophila Rhythmically Expressed Gene-5 (Dreg-5)

Van Gelder and others *(81)* reported 20 cycling genes in wild-type fly heads, and one of them *(Dreg-5)* was further characterized in detail *(82). Dreg-5* mRNA levels oscillate in phase with *per* in wild-type, and are constant

at its peak level in per^0 mutant background. These results indicate that *Dreg-5* is repressed directly or indirectly by *per.*

According to the author's recent BLAST search, the putative DREG-5 protein does not share any structural homology with known proteins. However, using a sequence analysis software (iPSORT: www.HypothesisCreator.net/iPSORT/), a putative *N*-terminal signal peptide was found, which indicates that DREG-5 may be a secretory protein. The functions of DREG-5 with respect to behavioral rhythms has not been determined due to a lack of mutation in this gene.

Circadianly Regulated Gene (Crg-1)

Temporal *crg-1* mRNA fluctuations are in phase with that of *per* mRNA in wild-type, and are abolished in per^0 and tim^0 mutants *(83).* The putative CRG-1 proteins show a marginal sequence homology with HNF3/fork head transcription factor family. Since *crg-1* mutant is not available, its rhythm-relevant functions have not been attributed to the *crg-1.*

Concluding Remarks

In addition to the genes listed above, recent genome-wide microarray screens have revealed a number of genes that are putatively regulated by central-clock functions *(51,84).*) Depending on the types of regulation, these genes have been grouped into three categories. The first group includes a small number of genes whose mRNA abundance fluctuates in a circadian manner, which is mediated "directly" by dCLK. For instance, *vri* and *pdp1* belong to this group, as previously discussed. The second group includes genes whose transcript levels oscillate in a circadian manner, but whose cyclings are likely to be regulated "indirectly" by dCLK (e.g., *takeout*). The final group of genes is featured by non-cycling with altered levels in $dClk^{Jrk}$ mutant background, indicating that these genes are also controlled "indirectly" by dCLK. *pdf* is clearly a member of this group *(23);* other examples are genes encoding odorant-binding proteins and antibacterial proteins *(51).*

Since only a small fraction of genes are under the direct regulation by *dClk,* and some of these genes encode transcription factors (e.g., *vri, pdp1, crg-1,*) it is conceivable that the indirect regulation of genes by *dClk* can be accomplished by these *dClk*-regulated secondary transcription factors. Such a transcriptional pyramid will eventually enable the *dClk* gene to control a large number of downstream genes, that in turn mediate separate clock-output pathways. Many of these output phenotypes are unknown, although recent studies have revealed new circadian phenotypes such as olfactory sensitivity *(85)* and female mating activity *(86).* On the other hand, some of these clock target genes may have nothing to do with circadian phenotypes, as clock genes also regulate non-circadian phenotypes such as cocaine-sensitization *(87)* and courtship song of male flies *(88),* indicating pleiotropic roles played by clock genes.

Finally, considering a number of genes and their functions discovered by the aforementioned microarray screens, one can imagine the impact of biological clock system on diverse physiological processes via controlling independent output pathways. Given the fundamental similarities of central clockworks between flies and mammals, but more complicated genomic organizations and physiological processes in mammals than those in flies, it is almost certain that even more genes are regulated by clock factors in mammals including humans. Clinically, perturbation of the principal oscillator functions would affect several output pathways, and a combination of these subnormal output factors might cause a particular behavioral problem (e.g., familial advanced sleep phase syndrome; *89*). Therefore, molecular genetic characterizations of individual clock-regulated genes will potentially provide important clues for adequate treatment of a specific rhythm-related disorder.

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